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# A determining influence for CpG dinucleotides on nucleosome positioning *in vitro*

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## ABSTRACT

DNA sequence information that directs the translational positioning of nucleosomes can be attenuated by cytosine methylation when a short run of CpG dinucleotides is located close to the dyad axis of the nucleosome. Here, we show that point mutations introduced to re-pattern methylation at the (CpG)<sub>3</sub> element in the chicken  $\beta^A$ -globin promoter sequence themselves strongly influenced nucleosome formation in reconstituted chromatin. The disruptive effect of cytosine methylation on nucleosome formation was found to be determined by the sequence context of CpG dinucleotides, not just their location in the positioning sequence. Additional mutations indicated that methylation can also promote the occupation of certain nucleosome positions. DNase I analysis demonstrated that these genetic and epigenetic modifications altered the structural characteristics of the (CpG)<sub>3</sub> element. Our findings support a proposal that the intrinsic structural properties of the DNA at the –1.5 site, as occupied by (CpG)<sub>3</sub> in the nucleosome studied, can be decisive for nucleosome formation and stability, and that changes in anisotropic DNA bending or flexibility at this site explain why nucleosome positioning can be exquisitely sensitive to genetic and epigenetic modification of the DNA sequence.

## INTRODUCTION

Nucleosomes are directed to precise positions by signals in the underlying DNA sequence (1,2). For example, histone octamers reconstitute onto the chicken  $\beta^A$ -globin gene region in characteristic translational positions that differ by as much as 1000-fold in their affinity for the histone octamer (3). Nucleosome positioning signals are likely to reflect a combination of structural features that are not always immediately apparent from the primary nucleotide sequence (4). A nucleosome position will be favoured only if the intrinsic, sequence-determined

structural properties of its DNA can accommodate the conformational demands imposed by tight coiling around the histone core. Two important parameters in this process are bending and flexibility (in particular, flexibility towards curvature, or bendability) (5). Rigid sequences, such as long T-tracts, are disfavoured from stable inclusion within nucleosomes *in vitro* and *in vivo* (6,7), a property that is exploited in transcriptional regulation in yeast (8,9). Sharply bent DNA can perform a similar function through exclusion from the nucleosome (10); certain proteins also induce such bending upon binding and thereby influence nucleosome placement and transcription (11). Conversely, a ~10 bp periodic distribution of short G/C-rich and A/T-rich motifs can provide anisotropic flexibility or curvature that is compatible with the periodic major and minor groove compression in nucleosomal DNA. This aids folding around the histone core and is favourable to positioning in a particular rotational setting (12–16). Promoter DNA elements with an intrinsic smooth curvature may function to establish appropriate local chromatin architecture (17,18).

Nucleosomal DNA deviates at several locations from a smooth path around the histone core (19). At 1.5 helical turns either side of the dyad axis, the DNA is required to accommodate severe deformation in order to make effective contact with the H3–H4 tetramer, and these sites in the nucleosome are uniquely sensitive to singlet oxygen (19,20). This singular demand on the structural properties of the DNA sequence, which requires sharp bending with departure from ideal base stacking, is likely to influence the translational positioning of nucleosomes (21) and probably explains why sequence-determined localized sites of inherent distortion in the DNA can play a positive role in positioning nucleosomes (22).

Biochemical studies have shown that (CpG)<sub>3</sub> sequence elements within certain chicken, mouse and human regulatory sequences commonly occupy dyad-proximal positions in the nucleosome, or are excluded to the periphery (23,24). When located close to the dyad axis, cytosine methylation at this short run of CpG dinucleotides is associated with nucleosome disruption in reconstituted chromatin (23,24). In the present study, we aimed to resolve the role of (CpG)<sub>3</sub> and its epigenetic modification in the translational positioning of nucleosomes. Our findings demonstrate the profound influence upon nucleosome formation of changes in sequence and methylation

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pattern in this short stretch of DNA sequence, when located at the  $-1.5$  site, can indeed exert a profound influence upon nucleosome formation.

## MATERIALS AND METHODS

### Mutagenesis

Plasmid pCBALE (3) comprises a 606 bp PvuII fragment of the chicken  $\beta^A$ -globin gene ( $-406$  to  $+200$ , relative to the cap site) cloned into the EcoRV site of pBluescript KS $^-$  (Stratagene). Point mutations were introduced into the promoter sequence by a two-stage PCR strategy (see Figure 1). First, Vent DNA polymerase (NEB) was used to amplify between the T3 primer and any one of a series of mismatched primers encompassing the (CpG) $_3$  at  $-295$  to  $-300$  (Trip1, 5'-CACAGCGCGGCCAGGCTGG-3'; Trip2, 5'-GCACAGCGGCCGCCAGGC-3'; Trip3, 5'-GAGCACAGGCCGCGCAGG-3'; Trip23, 5'-GCACAGCGGGCGCCAGGC-3'; Trip25, 5'-GCACAGCGCCGCCAGGC-3') or the CpG at  $-110$  (MonoA, 5'-GGCACCGCGCGGGAGGGAACG-3'; MonoB, 5'-GGCACCCCGCGCGAGGGAACG-3'), to give products of 190 or 380 bp, respectively. The PCR products were purified from a 3% NuSieve 3:1 agarose (FMC) gel and used in a second PCR. Amplification of pCBALE between any one of the primary PCR products and the M13-20 primer generated 790 bp products that were digested with XbaI and XhoI and cloned into XbaI/XhoI-cut pBluescript KS $^-$ .

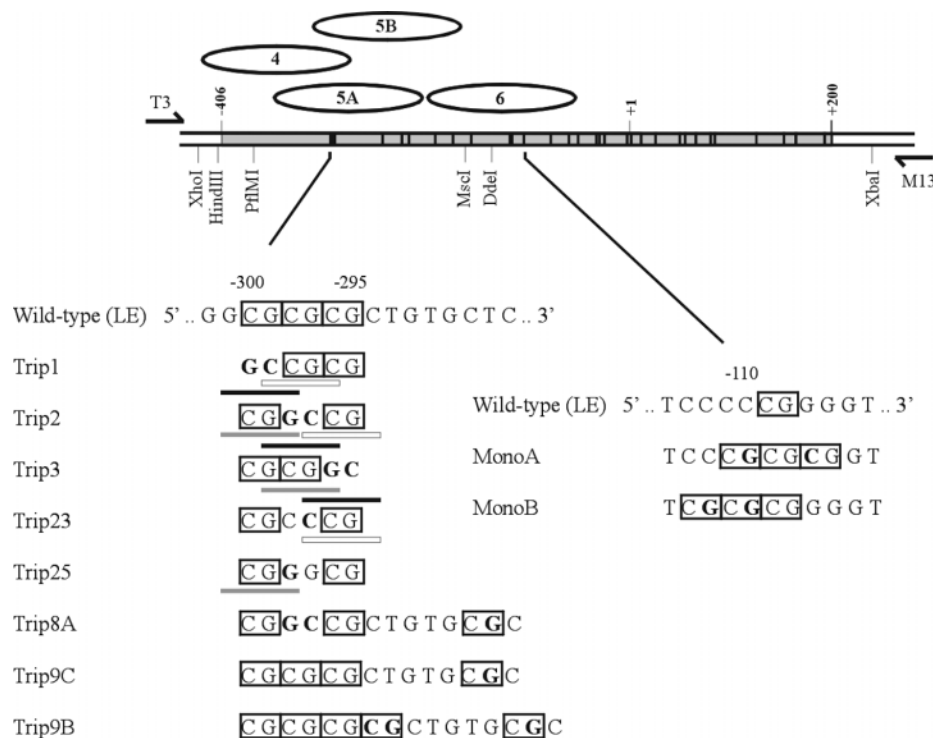
*Escherichia coli* JM110 (*dam dcm*) transformants were screened by sequencing the globin insert in its entirety. Trip8A contained a novel CpG dinucleotide created by a T  $\rightarrow$  G mutation that must have derived from a sequence variant of the Trip2 primer. Trip9B and 9C were generated when this point mutation was inserted into BssHII-cut pCBALE in order to place this novel CpG within a wild-type sequence context. Plasmids were transformed into *E. coli* DH11S for the production of single-stranded DNA which was generated by helper phage superinfection and isolated using the QIAprep Spin M13 kit (Qiagen).

### DNA methylation

Plasmids were linearized with AlwNI and then methylated with SssI methylase (NEB) until refractory to digestion by BstUI, HpaII, HhaI and AclI; in addition, BssHII, FspI, NotI and EagI were used as appropriate to the sequence mutation. Control reactions omitted the methylase.

### Chromatin reconstitution and mapping nucleosome positions by monomer extension

Procedures were essentially as described previously (25). Briefly, plasmid DNAs were reconstituted at a low histone/DNA ratio [0.4:1 (w/w) chicken erythrocyte core histones:DNA] to avoid neighbouring nucleosome interactions (26), by dialysis from high salt. Reconstitutes were digested with micrococcal nuclease (10 U/ml; Worthington) for 35 min at 0°C followed by 2 min at 37°C and the core particle DNAs



**Figure 1.** Mutagenesis of the chicken  $\beta^A$ -globin gene promoter sequence in pCBALE. The globin sequence (shaded) is numbered with respect to the cap site of the gene. The short bars are CpG dinucleotides. The ovals depict dominant positions adopted by histone octamers in reconstituted chromatin (3): nucleosomes 4 ( $-427$  to  $-281$ ), 5A ( $-354$  to  $-208$ ), 5B ( $-318$  to  $-172$ ) and 6 ( $-206$  to  $-62$ ). Point mutations (boldface) and CpG dinucleotides (boxed) are indicated for the sense (Watson) strand. Note how the Trip1 and Trip3 sequences, and the Trip23 and Trip25 sequences, are complementary for the 8 bp,  $-301$  to  $-294$ , whereas Trip2 is palindromic. Common tetranucleotide sequences are underlined: white, CCGC; grey, GCGG; black, GGCC. The T3 promoter and M13-20 oligonucleotide primers were used in the PCR mutagenesis.

(~146 bp) were isolated from a 3% NuSieve 3:1 agarose gel. The further isolation of core particle DNAs from denaturing (8M urea) 6% polyacrylamide gels did not change the results, indicating that internal nicking was not a significant factor. The 5' end-labelled core particle DNAs were annealed to excess single-stranded plasmid DNA and extended by Klenow DNA polymerase (NEB) in the presence and absence of XbaI. Purified extension products were analysed by denaturing 6% (19:1) PAGE followed by autoradiography and phosphorimaging. Phosphor images were analysed using Aida software (Fuji).

### Native polyacrylamide gel analysis of positioned nucleosomes

Wild-type and mutant plasmids were linearized with HindIII, dephosphorylated, then 5' end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase. Restriction with MscI released a 245 bp HindIII–MscI fragment of the globin promoter (Figure 1). The purified digest was reconstituted into chromatin by dialysis from high salt in the presence of unlabelled competitor DNA [207 bp sea-urchin 5S rDNA fragment (27)], at a chicken erythrocyte core histone to (total) DNA ratio of 0.65:1 (w/w). Positioning isomers were separated at 4°C in a 5% native polyacrylamide gel according to Meersseman *et al.* (27), followed by autoradiography and phosphorimaging.

### DNase I analysis of DNA structure

A 270 bp HindIII–DdeI fragment (Figure 1) was isolated from pCBALE and derivatives. Methylated and mock-methylated fragments were 5' end-labelled as described above, then cut with either MscI or PflMI to leave the sense (Watson) or antisense (Crick) strand labelled. An aliquot of 50–100 ng of the fragment was mixed with 750 ng of unlabelled carrier DNA and mildly digested in 50  $\mu$ l buffer (50 mM Tris pH 7.5, 1 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 0.1 mM DTT and 0.1 mg/ml BSA) with 0.05 U/ml DNase I for 2 min at 20°C. Reactions were stopped with 5 mM EDTA, 0.2% SDS, purified by phenol extraction and ethanol precipitation and then analysed by denaturing 8% (19:1) PAGE followed by autoradiography and phosphorimaging.

## RESULTS

### Point mutations at (CpG)<sub>3</sub> influence nucleosome positioning and its sensitivity to cytosine methylation

In reconstituted chromatin, the (CpG)<sub>3</sub> element within the promoter sequence of the chicken  $\beta^A$ -globin gene occupies a position –1.5 helical turns from the dyad axis of a positioned nucleosome (nucleosome 5A), with the minor groove facing the histone core (23). Point mutations were introduced to sequentially disrupt the methylation pattern at these CpG dinucleotides (Figure 1). Each CpG was eliminated in turn as a methylation site by CG  $\rightarrow$  GC transversion (mutations Trip1, Trip2 and Trip3). The central dinucleotide was also subject to single G  $\rightarrow$  C and C  $\rightarrow$  G changes (Trip23 and Trip25). Mutations maintained the GC content so as not to disrupt or introduce any positioning information in the form of a periodic distribution of short A/T and G/C sequence motifs. Note that the Trip1 and Trip3 sequences, and the Trip23 and Trip25

sequences, are complementary at the mutation site, while Trip2 is palindromic (Figure 1). The Trip8A mutant, which was recovered whilst screening the Trip2 clones, was found to contain a novel CpG dinucleotide which would occupy the –0.5 position of nucleosome 5A. The process of rescuing this novel CpG into a wild-type context to create Trip9C also created the (CpG)<sub>4</sub> of Trip9B. Mutations MonoA and MonoB introduced (CpG)<sub>3</sub> elements through minimal sequence changes at a location outside of nucleosome 5A, with the intention of determining whether these would reposition nucleosomes.

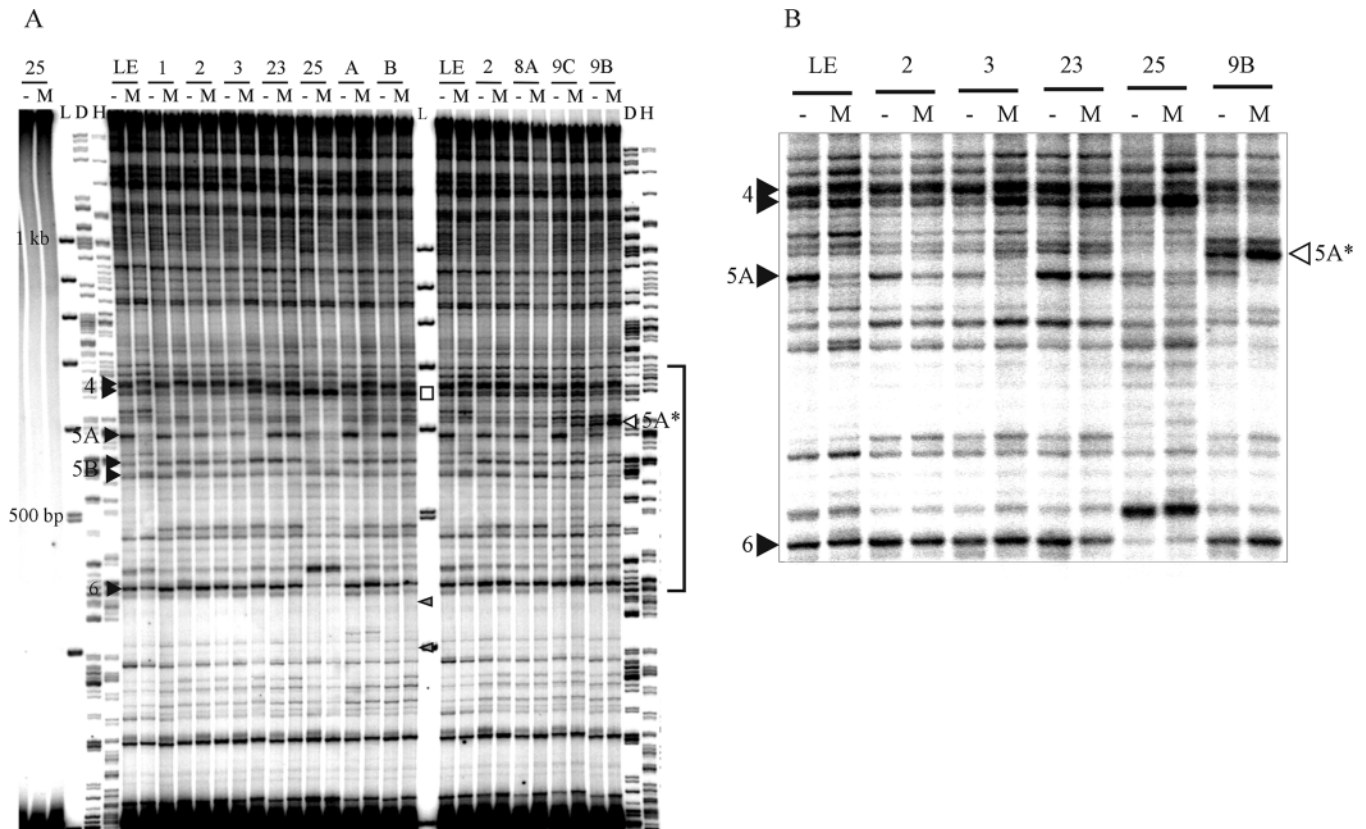
The wild-type (referred to as LE) and mutant plasmids were linearized, methylated at every CpG and then assembled into chromatin *in vitro*. Histone octamer positioning throughout the  $\beta^A$ -globin promoter sequence was assessed by monomer extension (26), a primer-extension technique that maps the boundaries of positioned nucleosomes at high resolution relative to unique restriction sites. The monomer extension products obtained for the wild-type and mutant promoter DNAs in methylated and unmethylated forms are shown in Figure 2. Each band in the analysis represents an upstream nucleosome boundary (mapped relative to the XbaI site, Figure 1). The variety in size and intensity of these bands reflects the fact that when limiting amounts of core histone octamers are reconstituted onto the  $\beta^A$ -globin promoter DNA, they are directed to numerous translational positions by signals of differing affinity.

In agreement with our previous findings, nucleosome positioning at one of the stronger sites, position 5A, was severely disrupted when the wild-type sequence was methylated [Figure 2, compare the (–) and (M) reactions of LE at 5A]. Methylation at the (CpG)<sub>3</sub> element is responsible for this behaviour (23). As summarized in Table 1 and discussed below, the point mutations introduced into (CpG)<sub>3</sub> had widely differing consequences for the formation of this nucleosome. Furthermore, these sequence changes altered the degree to which cytosine methylation at the remaining CpGs inhibited nucleosome formation.

Changing the upstream dinucleotide of (CpG)<sub>3</sub> into GC (mutation Trip1) had no discernable effect upon occupation of the 5A positioning site, whereas mutation of the central dinucleotide (Trip2) or, even more so, of the downstream dinucleotide (Trip3), significantly weakened the 5A nucleosome boundary [Figure 2A, compare the 5A band in the (–) lanes of LE, Trip1, Trip2 and Trip3]. Formation of the 5A nucleosome therefore appears strongly influenced by the presence of the central and, especially, of the downstream CpG of (CpG)<sub>3</sub>.

Methylation inhibited nucleosome positioning in each of these three mutants [Figure 2A, compare the 5A band in the (M) versus (–) lanes for LE, Trip1, Trip2 and Trip3]. Despite the overriding influence of the downstream CpG on the establishment of the 5A position, it is the methylation at the upstream and central CpGs that contributes most to nucleosome disruption. When either was methylated in combination with the downstream CpG (Trip2, Trip1), nucleosome disruption was not as effective as when they were both methylated (Trip3), and only in this latter circumstance was nucleosome disruption as effective as the wild type (Figure 2A, Table 1). When expressed relative to each unmethylated sequence, methylation in fact seemed to have a similar effect for Trip1, Trip2 and Trip3, causing an estimated 50–70% (estimated at 80% for the wild type) reduction in the strength of the





**Figure 2.** Monomer extension analysis of the effect of point mutation and cytosine methylation on histone octamer positioning by the globin gene promoter sequence. (A) Core particle DNAs were prepared from chromatin reconstitutes of mock (–) or CpG-methylated (M) wild-type (LE), Trip (1, 2, 3, 23, 25, 8A, 9B, 9C) and Mono (A, B) mutant plasmids. DNA polymerase extension was performed in the absence (the examples shown for Trip25 at the far left were representative of all such reactions) or presence of XbaI to map the upstream boundaries of positioned nucleosomes. The LE and Trip2 reactions are included twice for ease of comparison. The open square marks the junction of globin and vector sequences. Nucleosome boundaries are labelled as in Figure 1. The double banding at the nucleosome 4 and 5B boundaries may reflect a boundary effect from the (CpG)<sub>3</sub> or derivative sequence: the upper of the two bands labelled as nucleosome 4 marks a nucleosome (occupying –447 to –301) whose downstream boundary sits at this element; similarly, the lower of the two bands labelled as 5B marks a nucleosome (occupying –303 to –157) whose upstream boundary sits at this element. The shaded arrowheads indicate where a boundary would be expected in the MonoA and MonoB reactions if their new (CpG)<sub>3</sub> sequences directed nucleosome placement by adoption of the –1.5 or +1.5 position. Size standards comprised a 100 bp-step ladder (L; Promega) and HinfI (H) and DdeI (D) digests of phage λ. (B) Selected reactions were repeated using LE as the single-stranded DNA template throughout [rather than the single-stranded DNAs of each individual mutant, as used in (A)] with the same result. A magnified region from this analysis is shown, corresponding to the region of gel (A) indicated by the bracket.

**Table 1.** Point mutation and cytosine methylation at (CpG)<sub>3</sub> influence occupation of the 5A nucleosome position

Mutation	Non-methylated	Methylated
Wild type (LE)	+++	–
Trip1	+++	+
Trip2	++	+
Trip3	+	–
Trip23	+++	++
Trip25	+	–
Trip8A	++	–
Trip9C	+++	–
Trip9B	+	–

The grading is relative to the strength of the 5A nucleosome boundary in the wild-type, defined as reduced from (+++) to (–) by methylation.

5A boundary. That the consequences of epigenetic modification must be interpreted in the context of differences in the underlying sequence was underlined by additional mutations at the central CpG.

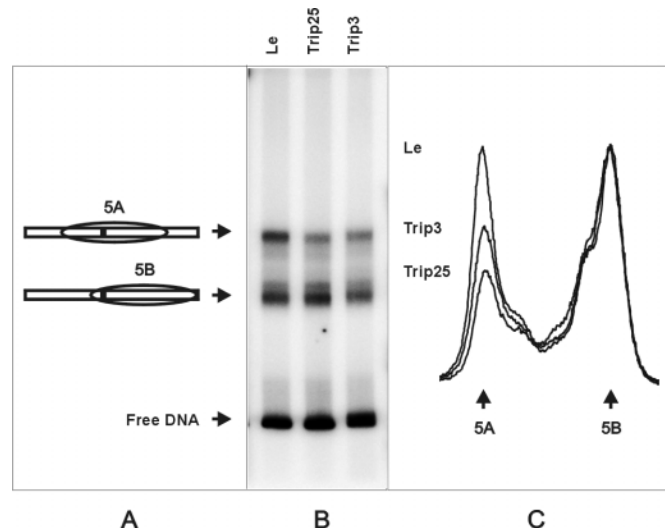
Changing single nucleotides at the centre of (CpG)<sub>3</sub> (Trip25 and Trip23, Figure 1) had contrasting consequences. Trip25 (like Trip3) strongly inhibited the formation of nucleosome 5A, whereas Trip23 (like Trip1) had no discernable effect [Figure 2B, compare the (–) reactions of LE, Trip23 and Trip25 at 5A]. Moreover, while methylation of Trip25 had a similar inhibitory effect to all the other mutants (an ~60% reduction in positioning relative to the unmethylated sequence), the Trip23 mutation almost abolished (no more than 15% relative inhibition) the capacity of methylation to disrupt the 5A nucleosome [Figure 2B, compare the 5A band in the (M) lanes of LE, Trip23 and Trip25]. Trip25, Trip23 and Trip2 have the same cytosine methylation sites, indicating that it is the local sequence context which determines their capacity for nucleosome disruption.

The Trip8A, Trip9B and Trip9C mutants introduced a novel CpG about one helical turn from (CpG)<sub>3</sub> closer to the dyad axis of nucleosome 5A; Trip8A and Trip9C were otherwise identical in sequence to Trip2 and wild-type LE,

respectively (Figure 1). This mutation at  $-0.5$  in itself had no discernable effect upon occupation of the 5A site [Figure 2A, compare the (–) lanes of Trip9C and LE, and of Trip8A and Trip2]. However, methylation at this novel CpG enhanced disruption of nucleosome 5A, while at the same time strengthening positioning at other local sites, in particular, at a site 15 bp upstream of 5A denoted 5A\* [Figure 2A, compare the (M) reactions of LE or Trip2 with those of Trip9C or Trip8A at 5A and 5A\*]. The 5A and 5A\* positions are predicted to be of opposite rotational setting: in 5A\*, the (CpG)<sub>3</sub> element (or derivative) has shifted from the  $-1.5$  site to the dyad axis, with the novel CpG shifted from  $-0.5$  to  $+1$ , both now facing away from the histone core (the precise rotational setting of nucleosome 5A\* needs to be confirmed experimentally to substantiate this point). Occupation of the 5A\* site was most pronounced for the methylated (CpG)<sub>4</sub> mutant, Trip9B, but here, even in the absence of methylation, nucleosomes occupied the 5A\* position in preference to 5A [Figure 2B, compare the (–) and (M) lanes of Trip9B at 5A and 5A\*]. A comparison of Trip9B with Trip9C [(CpG)<sub>4</sub> versus (CpG)<sub>3</sub>, Figure 1] in fact suggests that (in combination with the  $-0.5$  CpG) (CpG)<sub>4</sub> is intrinsically disfavoured from occupation of the  $-1.5$  site and, especially when methylated, is favourably accommodated at the nucleosome dyad. Thus, not only did sequence alterations within (CpG)<sub>3</sub> influence the formation of nucleosome 5A, but also the addition of an extra CpG to this element promoted repositioning.

When (CpG)<sub>3</sub> sequences were inserted at two locations within C<sub>5</sub>G<sub>4</sub> (mutations MonoA and MonoB, Figure 1), new nucleosome boundaries did not emerge on the globin promoter at locations that would have reflected occupation by these (CpG)<sub>3</sub> elements of  $\pm 1.5$  positions in the nucleosome (Figure 2A, compare the MonoA and MonoB reactions with LE at the shaded arrowheads). The only consequence of these sequence changes was a new, but very weak and methylation-independent position established between the ‘predicted’ locations on the MonoA sequence (Figure 2A, between the shaded arrowheads). In this nucleosome, the introduced (CpG)<sub>3</sub> element is centered  $\sim 5$  bp upstream of the dyad axis. A (CpG)<sub>3</sub> element does not therefore constitute a ‘dominant’ positioning element in itself, underlining the conclusion that the mutation and methylation effects described above were mediated at CpG dinucleotides of an appropriate location and natural sequence context within the 5A position.

We note that the influence of these various genetic and epigenetic modifications was not restricted to nucleosome 5A. Local overlapping and flanking positions were also affected, including the relative intensity of the doublet boundary band of the strong, mutually-exclusive nucleosome 4. This may in part reflect a ‘redistribution’ of nucleosomes from a disfavoured 5A position (although not in the case of Trip23). For the Trip25 reconstitute, there was a methylation-independent 10 bp shift in the nucleosome 6 boundary that could reflect a readjustment of positioning between nucleosomes 4 and 6 in response to the effective absence of nucleosome 5A (and, largely, also of 5B); the boundaries of nucleosomes 5A and 6 would normally abut each other, if occupying the same DNA molecule (Figure 1).



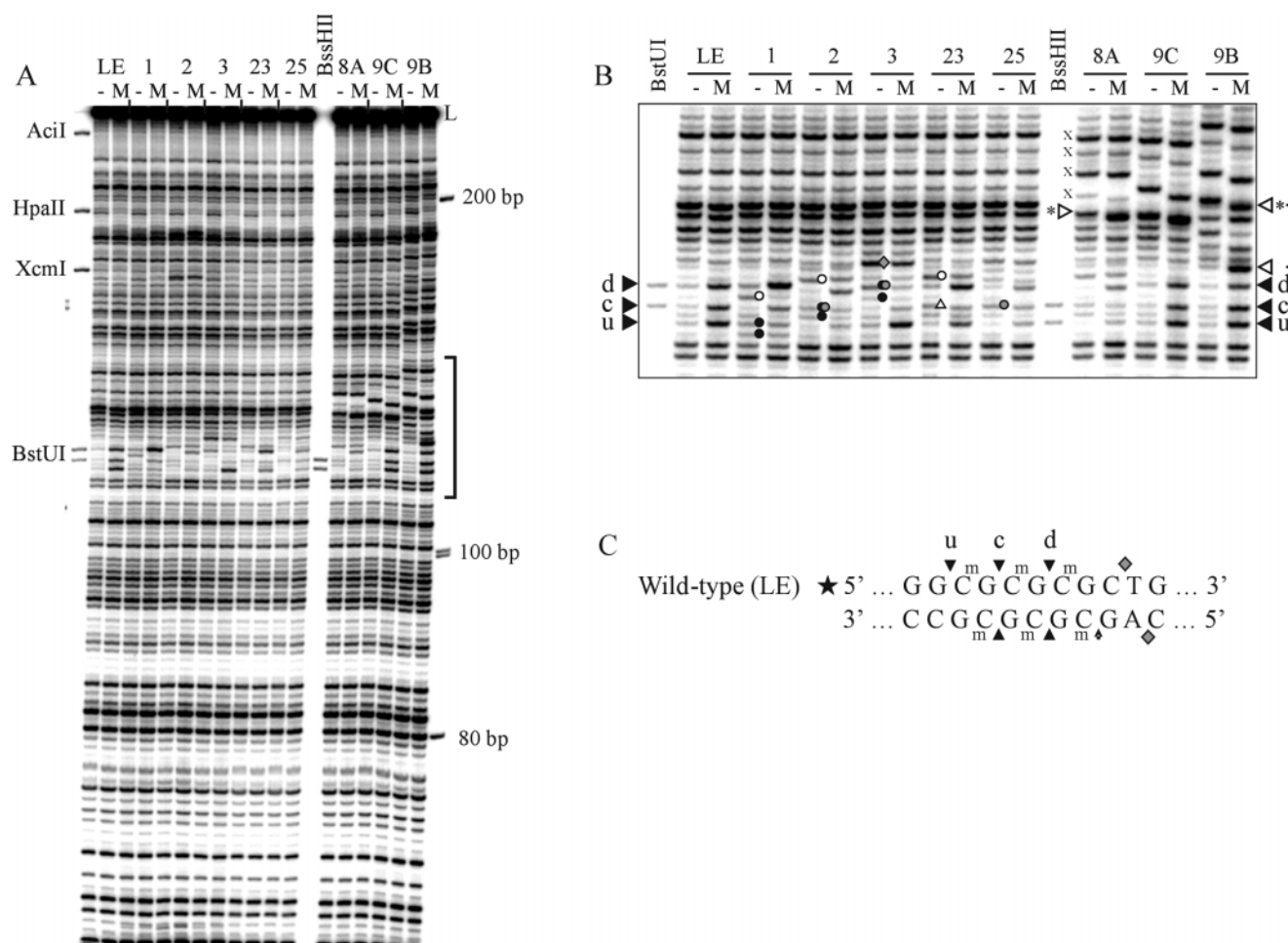
**Figure 3.** Native polyacrylamide gel analysis revealed that point mutations at (CpG)<sub>3</sub> inhibit the formation of nucleosome 5A. (A) Nucleosomes were reconstituted onto an unmethylated restriction fragment of the globin promoter encompassing the (CpG)<sub>3</sub> element or derivative (black bar). (B) Reconstitutes were electrophoresed in a native 5% polyacrylamide gel to separate the near centrally located 5A nucleosomes from the end-positioned 5B nucleosomes. (C) Phosphorimager traces of the lanes in gel (B), normalized to the peak labelled 5B (which may also comprise uncharacterized end positions).

### Sequence mutations at (CpG)<sub>3</sub> effect nucleosome exclusion

Reduced nucleosome stability could be reflected in a low frequency of occupation of the positioning site and/or in an enhanced susceptibility to the nuclease employed to prepare core particle DNA. We re-examined the results for Trip3 and Trip25 using a technique that avoids nuclease digestion in the characterization of reconstituted chromatin. Nucleosomes were reconstituted onto a short restriction fragment that incorporates the 5A positioning site centrally (Figure 3A), enabling its occupation to be visualized directly, as this complex constitutes the slowest-migrating positioning isomer in a native polyacrylamide gel [Figure 3B; (23)]. It can be seen that both Trip25 and Trip3 inhibited *per se* the formation of the 5A nucleosome, by  $\sim 50$  and  $30\%$ , respectively, relative to wild type (Figure 3C). While this is in reasonable quantitative agreement with the monomer extension data, we cannot entirely exclude the possibility of enhanced nuclease susceptibility as a further manifestation of the weakened affinity of the nucleosome for the 5A position.

### Genetic and epigenetic modification alter the structural properties of (CpG)<sub>3</sub>

The experiments described above showed that minimal changes in the nucleotide sequence could effect a dramatic change in the capacity for DNA incorporation into the nucleosome. Furthermore, the local sequence context, and not just the presence of CpG dinucleotides *per se*, determined the capacity of methylation to inhibit the formation of nucleosome 5A. Mild DNase I digestion was undertaken to see whether sequence-determined DNA structure could underlie these



**Figure 4.** DNase I analysis revealed that the structural properties of  $(\text{CpG})_3$  are altered by point mutation and cytosine methylation. Panel (B) shows a magnified region (equivalent to the bracket) from a longer-run version of the gel shown in (A). A restriction fragment of the globin promoter, labelled at the upstream end of the sense strand [star in (C)], was mildly digested with DNase I in methylated (M) and mock-methylated (–) form. Closed arrowheads in (B) indicate DNase I cleavage sites [upstream (u), central (c) and downstream (d)] enhanced by methylation of the wild-type  $(\text{CpG})_3$ ; those on the antisense strand (Figure S1) are also marked in (C), with the smaller arrowhead indicating weak enhancement. Cleavage sites in the unmethylated DNAs associated with particular sequence contexts are marked: an open circle indicates cleavage at  $\text{CC}^\nabla\text{GC}$ ; a shaded circle at  $\text{GCG}^\nabla\text{G}$ ; closed circles at  $^\nabla\text{G}^\nabla\text{GCC}$ ; an open triangle at  $\text{G}^\nabla\text{CCC}$ ; the shaded diamond marks cleavage specific to Trip3, as indicated (on the wild-type sequence) in (C). White arrowheads indicate nuclease cleavage on the 5' side of the cytosine at an additional CpG of  $(\text{CpG})_4$  in Trip9B [no asterisk; as a 2 bp insertion within  $(\text{CpG})_3$ , it is somewhat arbitrary which of these four CpGs is arrowed], and at the novel CpG in Trip8A, Trip9B and Trip9C (arrowhead with asterisk). The dashed line spans a methylation-modulated structure in Trip9B. Bands are marked (x) to exemplify the compression in Trip8A, Trip9B and Trip9C. Size standards comprised the 100 bp step ladder (L) and restriction fragments that locate some CpG dinucleotides; the cleavage sites for BssHII coincide with (u) and (c), while those for BstUI coincide with (c) and (d).

observations. This approach indeed revealed a complexity of differences in the structure of  $(\text{CpG})_3$  and its derivatives, and a further sequence context-dependent switching in structure in response to epigenetic modification.

The  $(\text{CpG})_3$  element was unreactive to DNase I [Figure 4A, LE lane (–);  $(\text{CpG})_3$  is located by alignment with the BstUI and BssHII marker fragments]. Upon methylation, nuclease cleavage was strongly enhanced on the 5' side of each cytosine of  $(\text{CpG})_3$  on the sense strand [Figure 4B, compare cleavage at (u), (c) and (d) in LE lanes (–) and (M)]. Methylation-enhanced cleavage was also seen on the antisense strand [Figure S1; (23)]. This methylation-determined cleavage pattern, which is summarized in Figure 4C (see also Figure S2), is consistent with a widening of the minor groove (28) where it faces the histone octamer, and might well be incompatible

with the direction of the bending required of the DNA at the –1.5 site for stable incorporation into the 5A nucleosome.

The point mutations altered the DNase I attack pattern in a manner that could be correlated to sequence context. Cleavage within the tetranucleotide  $\text{CC}^\nabla\text{GC}$  (open circle, Figure 4B; Figure S1) represented one of the strongest features in the unmethylated mutant DNAs. Cleavage was also pronounced within the complementary sequence  $\text{GCG}^\nabla\text{G}$  (shaded circle, Figure 4B, Figure S1). The  $\text{CCGC}^\nabla\text{GCGG}$  tetranucleotide, which is present in all of the  $(\text{CpG})_3$  mutants (Figure 1), therefore has distinct structural properties and may adopt a particular minor groove structure.

The tetranucleotide  $^\nabla\text{G}^\nabla\text{GCC}$  was also cleaved characteristically by DNase I (the pairs of closed circles, Figure 4B), although cleavage was not so apparent on the antisense strand.



The nucleosome-excluding Trip3 sequence also demonstrated the strongest difference of all in cleavage of the mutant and wild-type sequences. This cleavage site (shaded diamond, Figure 4B and C; see also Figure S1) occurred in consequence of changing CGCT (LE) to GCCT.

For all mutants, the principal effect of methylation was to enhance nuclease cleavage at the 5' side of cytosines within CpG dinucleotides remaining from (CpG)<sub>3</sub> [i.e. at (u), (c) or (d), Figure 4B]. It is indeed notable that cleavage was specifically enhanced at methylated cytosines within (CpG)<sub>3</sub> and its derivatives [e.g. cleavage at the CpG within the HpaII site of the globin promoter was in fact weakened by methylation (Figure 4A); on the antisense strand, cleavage at this CpG was not altered by methylation]. The degree of cleavage was dependent upon the local sequence context. Thus, although the methylated Trip1 structure retained cleavage at sites (c) and (d) from the wild type, cleavage at (c) was in relative terms suppressed and cleavage at (d) was enhanced [Figure 4B, compare the (M) lanes of Trip1 and LE at (c) and (d); see also Figure S2]. The equivalent pattern was seen 'in reverse' when the downstream CpG was mutated [Figure 4B, compare the (M) lanes of Trip3 and LE at (c) and (u); see also Figure S2]. These same features were retained on the antisense strand of both mutants (Figure S1), suggesting that methylated Trip1 and Trip3 might adopt a similar, but reciprocally orientated structure.

Methylation enhanced the nuclease cleavage at the two remaining CpG dinucleotides of Trip2, Trip23 and Trip25 to very different degrees [Figure 4B, compare their (M) reactions at (u) and (d)]. This was again a consequence of local sequence context (i.e. these cleavage patterns were influenced by the differing sequence between the two CpGs). Although these different structural characteristics, as revealed by the intensity of cleavage at (u) and (d), are likely to underlie their distinct capacities for nucleosome incorporation (Table 1), it is not possible to simply correlate the two characteristics directly. It is methylated Trip23, which was still readily incorporated into the 5A nucleosome, whose cleavage pattern perhaps most closely resembles that of the methylated wild type. However, on the antisense strand it is the complementary Trip25 sequence that shows the stronger nuclease cleavage at the two methylated cytosines (Figure S1).

The novel CpG shared by Trip8A, Trip9C and Trip9B (the single nucleotide change that distinguishes Trip8A from Trip2, and Trip9C from wild-type LE, Figure 1) introduced local changes into the nuclease cleavage pattern that were, furthermore, strongly enhanced by methylation (Figure 4B, at and just below the open arrowhead with asterisk; see also Figure S1). In Trip9B, the (CpG)<sub>4</sub> element (created by a 2 bp insertion that distinguishes Trip9B from Trip9C, Figure 1) adopted an extended form of the wild-type structure (Figure 4B, open arrowhead). It is notable that between the (CpG)<sub>4</sub> element and the novel CpG, the nuclease cleavage pattern of Trip9B was quite different from that of the same sequence of Trip8A and Trip9C (Figure 4B, dashed line). This cleavage pattern was furthermore substantially altered by methylation beyond the immediate context of the methylated cytosines [Figure 4B, compare the (–) and (M) reactions of Trip9B at the dashed line]. This methylation-modulated structure might well underlie the pronounced, methylation-enhanced adoption of the 5A\* position on the Trip9B sequence.

A region of the gel (some affected bands are marked 'x' in Figure 4B) was subject to a compression that was enhanced by methylation. Its cause could relate to the fact that the T → G mutation, which established the novel CpG (Figure 1), created a 14 bp palindrome centered on GCGC [the palindrome [RY(YR)<sub>5</sub>RY] begins at the last G of the (CpG)<sub>3</sub>, or derivative, and is therefore equivalent in the Trip8A, Trip9B and Trip9C sequences]. Whether these compressions in the denaturing gel are symptomatic of related structural distortions in the double-stranded DNA that could play a role in adoption of the 5A\* position remains uncertain, as the occurrence and magnitude of the compression did not (by comparison of Figures 2 and 4) correlate with occupation of the 5A\* position.

## DISCUSSION

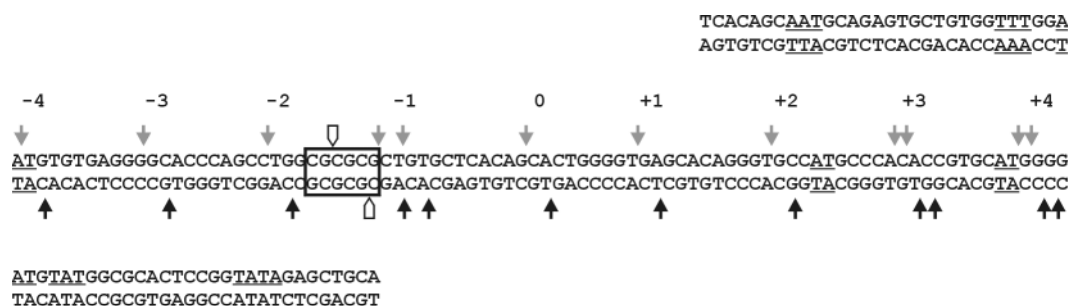
### The role of (CpG)<sub>3</sub> in nucleosome positioning

The short DNA sequence spanning the –1.5 site clearly has a disproportionate influence on the formation and stability of nucleosome 5A, and this role is better fulfilled by (CpG)<sub>3</sub> than a variety of non-alternating C/G combinations. This appears to support the findings of other mutation studies, including point mutation at a –1.5 site, suggesting a key role for such dyad-proximal sequences in the translational positioning of nucleosomes (22,29,30). However, our results demonstrate that (CpG)<sub>3</sub> does not constitute a positioning signal in itself. It is therefore possible that the genetic and epigenetic changes at (CpG)<sub>3</sub> affect its compatibility with a 'principal' positioning signal located elsewhere in the nucleosome. A prime candidate would be the sequence occupying the (arguably) symmetrical +1.5 histone-contact site. We did not observe a strong nucleosome position incorporating the (CpG)<sub>3</sub> at its +1.5 site. Others have demonstrated that A/T-rich sequences, and the TA step in particular, are strongly favoured at the +/–1.5 sites of some positioning sequences, where they may impart localized flexibility or deformability [because of the low stacking energy of WW steps (31)], or inherent curvature or distortion in the DNA (22,32,33). However, a dominant A/T-rich +1.5 positioning signal can certainly be discounted for the 5A nucleosome (Figure 5). The positioning signal might be widely distributed throughout the 5A nucleosome, and may equally be sensitive to the structure of the DNA bridging between DNA-histone contact sites (34). Although such additional DNA features must determine the formation of nucleosome 5A, their contribution could, nevertheless, be overridden by genetic and epigenetic changes at the –1.5 site, and this is likely to reflect sequence (in)compatibility with structural requirements at this specific site in the nucleosome structure.

### Structural characteristics of (CpG)<sub>3</sub> and derivative sequences

The key parameter is the energy requirement for deformation of the sequence into the sharply bent structure at the –1.5 site in the nucleosome. Intrinsic structure and flexibility (bending and twisting potential) are determined by the sequence-dependent conformational preferences of each base step and are influenced by the exocyclic groups of the bases (5), both of which have been altered experimentally in the present study.





**Figure 5.** (CpG)<sub>3</sub> spans the  $-1.5$  site in nucleosome 5A. DNase I cleavage sites (arrows) for the antisense (Crick) strand of the nucleosomal DNA are from Davey *et al.* (23) and are predicted for the sense (Watson) strand. The locations of the phosphates (open arrows) that are attached to the histones in the minor groove are deduced from the NCP147 nucleosome (4). The underlining makes clear the scarcity and peripheral location (absent altogether from the central 40 bp) of WW steps in this positioning sequence.

The accepted view is that CG (and GC) steps are very stable (compared to TA steps) because the base stacking requirements are optimized (31). The introduction of out-of-alternation base steps into (CpG)<sub>3</sub> is predicted to reduce this stability and, all else being equal, to reduce the energy required for deformation. The results we have obtained for the (CpG)<sub>3</sub> sequence and its derivatives appear contrary to these expectations. However, there are alternative perspectives suggesting a more flexible nature to CG steps (35,36). When calculated according to Packer *et al.* (37), who argue that the conformations and flexibilities of the CG, GC and CC/GG steps are especially influenced by the conformational coupling with neighbouring steps, the alternating YR structure of (CpG)<sub>3</sub> is predicted to be comparatively flexible. Furthermore, this flexibility is reduced by the sequence changes we introduced (Figure S3), which is in keeping with the fact that, where affected, nucleosome formation was always inhibited, never enhanced, by deviation from the wild-type sequence. Isotropic flexibility cannot, however, explain why mutations at the same CpG dinucleotide in Trip2, Trip23 and Trip25 behave so differently regarding their capacity for nucleosome incorporation (see Figure S3).

The DNase I results suggest that the introduction of out-of-alternation base steps disrupts the structure of (CpG)<sub>3</sub>. The GG/CC step has been cited as particularly disruptive with regard to base stacking (38) and its introduction at the centre of (CpG)<sub>3</sub> resulted in enhanced nuclease cleavage (at CG<sup>∇</sup>CCCG) that was apparent in the sense strand of nucleosome-accommodating Trip23 and in the antisense strand of nucleosome-excluding Trip25 (open triangle, Figure 4B and Figure S1). Spanning the  $-1.5$  site, Trip23 and Trip25, and likewise Trip1 and Trip3, contain the same 8 bp sequence in opposing orientation (Figure 1), a characteristic that underlies these nuclease attack patterns and which points to a role for anisotropy in structure (or flexibility). Asymmetric cleavage within CC<sup>∇</sup>GC.GCG<sup>∇</sup>G indicated that a particular minor groove structure might be adopted within this tetranucleotide. Notably, both sequences that accommodate nucleosome 5A (Trip1 and Trip23) include this tetranucleotide in one orientation, whereas both sequences that inhibit nucleosome formation (Trip3 and Trip25) contain it in the opposing orientation (see Figure 1 and the open and shaded circles in Figures 4B and S1). The palindromic Trip2 sequence, which comprises one copy of this tetranucleotide in each orientation (and

therefore shows the open and shaded circles in both strands, Figures 4B and S1), lies between the two extremes with regard to its capacity for incorporation into the 5A nucleosome. The fact that the CCGC tetranucleotide occupies the same position in Trip1 and Trip3 (Figure 1) underlines the importance of sequence orientation. It is also apparent that only the shaded circles of Trip3 and Trip25 align with what are methylation-enhanced cleavage sites in the wild type [sites (c) and (d), Figure 4B] and, as such, could emulate features of that nucleosome-excluding DNA structure. However, this is equally the case on the antisense strand for the nucleosome-accommodating Trip1 and Trip23 sequences (Figure S1). Thus, if the behaviour of the sequence mutants towards the nucleosome is to be explained through emulation of features of the methylated wild-type structure, a strand asymmetry must be invoked (i.e. it cannot be assumed that all features in the methylated wild-type structure, as revealed by nuclease digestion, dictate the incompatibility with the  $-1.5$  position).

Alternatively, the CCGC.GCGG tetranucleotide could impart a localized anisotropic flexibility, specifically, the capacity to accommodate bending in a preferred direction that was compatible (Trip1 and Trip23), or (diametrically) incompatible (Trip3 and Trip25), with the DNA path at the  $-1.5$  site.

The tetranucleotide GGCC was also cleaved characteristically by DNase I. This structural feature 'moves through' the  $-1.5$  position from Trip1 to Trip2 to Trip3 (Figures 1 and 4B), mirroring the trend for reduced incorporation into the 5A nucleosome [i.e. Trip1 (=LE) > Trip2 > Trip3]. The (CpG)<sub>3</sub> element is not located centrally with respect to the phosphates likely to be involved in establishing the  $-1.5$  position (Figure 5). In contrast to Trip1, the nucleosome-disrupting Trip3 mutation targets this change in structure (or loss of flexibility; inflexibility being a particular characteristic of this tetranucleotide predicted from Figure S3) to the histone-binding, downstream end of the (CpG)<sub>3</sub> element. The strong, methylation-independent cleavage of Trip3 (Figure 4C, shaded diamond), which would coincide with the  $-1$  position, would also be proximal to this phosphate (Figure 5).

The structure of DNA in the nucleosome is markedly different from that of protein-free DNA (4), and parameters derived from structural studies of oligonucleotides and non-histone-DNA complexes may be unreliable predictors

in a nucleosomal context (4,39). At the  $-1.5$  site, the DNA must accommodate bending around the histone octamer as well as local distortion, and the latter may be influenced by the former, i.e. it may not be easy to predict how sequences in the curved state respond to further forces leading to distortion. The deformability we 'detect' in the  $(\text{CpG})_3$  motif could thereby be a function of nucleosomal context. The high shift propensity (a flexibility parameter employed by Packer *et al.*, see above) of the GC step has been cited in the context of accommodating the pronounced alternate shifting of the NCP147 DNA at the 0.5, 1.5 and 2.5 sites in the nucleosome crystal structure (4). The globin promoter sequence also includes a GC step at  $-0.5$  (GCGC for Trip8A, Trip9B and Trip9C) in addition to those at  $-1.5$  (Figure 5), and these base steps may likewise help to circumvent base clash in the minor groove at these locations in the 5A nucleosome. Perhaps,  $(\text{CpG})_3$  can accommodate severe bending because although it may not be especially flexible in free solution, in the context of the nucleosome structure, this sequence is able to avoid base clash at the  $-1.5$  site, until that is, methyl groups are added.

### Cytosine methylation modifies the sequence-dependent structural properties of DNA

The effect of cytosine methylation on the 5A nucleosome was determined not only by the position, but also by the sequence context of CpG dinucleotides. Methylation, therefore, modulates a structural character that is sequence-dependent, and its effect operates in the context of the sequence-determined structural parameters of each mutant. These underlying structural properties of bending and bendability may dictate compatibility with the path of the DNA at the  $-1.5$  site in spite of methylation, such that an inhibitory effect on formation of the 5A nucleosome may be largely absent (Trip23), or at least reduced (Trip1 versus wild type) for certain sequences.

Exocyclic groups such as 5-methyl cytosine can reduce backbone flexibility and dynamics (40–44), and this has been proposed as an explanation for our findings, and those of Buttinelli *et al.* (45), who also found that cytosine methylation reduces DNA affinity for the histone octamer and affects its positioning. An increase in stiffness at the  $-1.5$  site would fit with the general association between its methylation and a reduction in nucleosome affinity. The substantial effect of methylation on the 5A nucleosome may be explained by the fact that steric resistance to bending imposed by the methyl groups would be expected to have greatest effect where histone-induced groove compression, and thereby the potential for local steric clashes, is greatest (45).

If methylation increases DNA stiffness, then it might be predicted to increase the discrimination of nucleosome positioning (5,45). Loss of positioning at 5A would be one consequence of this, but the enhancement of alternative positions may be another [nucleosome 4; see also (24)]. On the Trip9B sequence, in particular, the balance between occupation of the alternative 5A and 5A\* nucleosome positions was shifted strongly in favour of the latter by methylation. The DNase I results suggest that the structural changes at (and spanning between) the novel CpG and the  $(\text{CpG})_4$  element (located at  $-0.5$  and  $-1.5$ , respectively, in 5A) appear to be responsible for promoting re-accommodation of the DNA at a rotational

setting where both would now face away from the histone core.

In summary, the biochemical experiments of the present study indicate that DNA methylation clearly influences structural properties of DNA pertinent to nucleosome formation. The consequences of methylation are revealed in a simple reduction in affinity for the histone octamer, or a more complex influence on the balance between the adoption of alternative positions. In all events, the occurrence and nature of such consequences for the nucleosome are critically determined by the primary DNA sequence. In view of the importance of promoter nucleosome placement in gene regulation, a conclusion of this work is that a favourable accommodation of CpG dinucleotides at the  $-1.5$  site creates a context in which epigenetic modification can have a decisive effect upon critical histone:DNA interactions resulting in nucleosome repositioning.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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